EXPERIMENTAL ANALYSIS OF A PATERNALLY INHERITED EXTRACHROMOSOMAL FACTOR

JOHN H. WERREN*[,1] AND JOHANNES VAN DEN ASSEM[†]

*Departments of Zoology and Entomology, University of Maryland, College Park, Maryland 20742, and [†]Zoologisch Laboratorium, Universiteit Te Leiden, RA 2300 Leiden, Holland

Manuscript received March 13, 1986
Revised copy accepted May 30, 1986

ABSTRACT

Virtually all known cases of extrachromosomal inheritance involve cytoplasmic inheritance through the maternal line. Recently, a *paternally* transmitted factor that causes the production of all-male families has been discovered in a parasitic wasp. The wasp has haplodiploid sex determination: male offspring are haploid and usually develop from unfertilized eggs, whereas females are diploid and usually develop from fertilized eggs. It has been postulated that this paternal sex-ratio factor (*psr*) is either (1) an infectious agent (a venereal disease) that is transmitted to the female reproductive tract during copulation with an infected male and, subsequently, causes all-male families or (2) a male cytoplasmic factor that is transmitted by sperm to eggs upon egg fertilization and, somehow, causes loss of the paternal set of chromosomes.—Experimental evidence is presented which shows that the factor requires egg fertilization for transmission to the next generation; therefore, it is likely to be a cytoplasmic factor. Significant potential intragenomic conflict results from the presence of this factor and two other sex-ratio distorters in this wasp species.

EXTRACHROMOSOMAL inheritance is widespread among plants and animals. The overwhelming majority of cases of extrachromosomal inheritance are cytoplasmic factors that are transmitted maternally through the egg cytoplasm. Examples are organelle inheritance, such as chloroplasts and mitochondria, and intracellular microorganisms, such as rickettsia, spiroplasms, viruses and others (COSMIDES and TOOBY 1981; EBERHARD 1980). Paternal extrachromosomal inheritance is extremely rare, and its virtual absence has generally been attributed to a relative deficiency of cytoplasm in sperm (or pollen) compared to eggs. Some authors have suggested that "genetic conflict" among cytoplasmically inherited factors has led to the suppression of paternal inheritance (BIRKY 1976; COSMIDES and TOOBY 1980). In either case, the general absence of this form of inheritance is of theoretical interest.

Recently, WERREN, SKINNER and CHARNOV (1981) discovered a paternally inherited extrachromosomal factor that causes the production of all-male broods in the parasitic wasp, *Nasonia vitripennis*. The factor, henceforth termed

[1] Present address: Department of Biology, University of Rochester, Rochester, New York 14627.

psr (paternal sex ratio), was shown to be extrachromosomally inherited by use of the haplodiploid sex determination of wasps. In most Hymenoptera (bees and wasps), males develop from unfertilized (haploid) eggs and females develop from fertilized (diploid) eggs; therefore, male progeny receive no chromosomal genes from their "father." WERREN, SKINNER and CHARNOV (1981) were able to show, by use of genetic markers, that *psr* males do not transmit chromosomal genes to their male progeny, but that they do transmit the *psr* trait. Hence, by definition, it is extrachromosomal. The existence of this paternal extrachromosomal factor raises several important questions. How is the factor transmitted to successive generations? Why does paternal inheritance occur in this biological system when it does not occur in others?

The paternal sex-ratio factor is one of three extrachromosomal sex-ratio distorters so far discovered in this parasitic wasp (SKINNER 1982, 1985; WERREN, SKINNER and CHARNOV 1981; WERREN, SKINNER and HUGER 1986). In addition to *psr*, *msr* (maternal sex ratio) is a maternally inherited factor that causes the production of approximately 97% female progeny in carrier females, and *sk* (sonkiller) is a maternally and infectiously transmitted bacteria that causes male egg lethality. Sex-ratio theory predicts that maternally inherited factors are selectively favored to bias sex ratio toward female progeny, since females are the only sex that can transmit a maternal factor to future generations. Female-biased sex ratios enhance transmission of maternal factors. For similar reasons, paternal factors should be selectively favored to bias sex ratios toward male progeny; this, indeed, is what *psr* does. The *psr* factor, therefore, is apparently an exception that proves the rule of sex-ratio theory, that sex-ratio distortion should be toward the sex through which an extrachromosomal factor is inherited. Studies of this unusual factor, therefore, will enhance understanding of both extrachromosomal inheritance and sex-ratio evolution.

Two possible modes of transmission for *psr* were postulated by WERREN, SKINNER and CHARNOV (1981). First, *psr* may be an infectious agent of the wasp's reproductive tract that is venereally transmitted from infected males to females. The agent could then be transmitted to eggs passing down the reproductive tract. If the infection interfered with normal fertilization processes, then all male broods would result. This is termed the "infection" hypothesis. The second possibility is that *psr* may be associated with the sperm of *psr* males and, thus, gain entry into the egg during fertilization. There, the *psr* would have to cause rejection of the paternal chromosomal genome so that the egg would remain haploid and male, because WERREN, SKINNER and CHARNOV (1981) found that *psr* males are haploid and derive their chromosomal complement maternally. This is termed the "fertilization" hypothesis. The *psr* agent could be loosely associated with sperm, such as in the seminal fluid, or it could be a cytoplasmic or transposable element within the sperm.

The purpose of this paper is to describe experiments that elucidate the inheritance pattern of this unusual extrachromosomal factor. Experimental evidence is presented which shows that the *psr* factor requires egg fertilization for transmission to the next generation, in support of the fertilization hypothesis.

MATERIALS AND METHODS

General: *N. vitripennis* is a small (~3 mm) chalcidoid wasp which parasitizes many species of cyclorrhaphous fly pupae. Its biology is relatively well studied (WHITING 1967).

Experiments were performed under constant light at 25°, and two different host species were used, either *Sarcophaga bullata* or *Calliphora vomitora.* Unless indicated otherwise, experiments utilized Calliphora hosts. Various genetic strains are available (SAUL *et al.* 1965). The following stocks were used. Wild-type stocks: Carolina Biological (cb^+) [= Ithaca, New York (Ith^+)], Leiden Lab II (Lab II); Other stocks: paternal sex ratio (*psr*), scarlet-eye (st-1), slbrr4 (*msr*) and an artificially selected high male sex-ratio stock (sel). Characteristics of these stocks are discussed in the relevant experiments. In standard experiments, single female wasps were placed in a test tube with several hosts for a specified time period. Progeny from the hosts were allowed to develop to adulthood and then were scored for the particular phenotype under study.

Assay for *psr*: The *psr* trait was routinely assayed by individually mating presumptive *psr* males to Lab II females and scoring the sex ratio of the resulting progeny. If a greater than 90% male brood results, then the male parent was scored as *psr*. This phenotypic test has a small bias since approximately 5% of control crosses also result in more than 90% male broods, due to inadequate mating. In certain cases of ambiguity, at least five males from the F_1 brood were also tested, and if two or more produced all-male broods, then the parent was determined to be *psr*.

Experiments: The first series of experiments were conducted to characterize the basic inheritance pattern of *psr*. These are as follows:

1. Total brood: an initial experiment to determine what proportion of the males from a *psr* brood inherit the trait.
2. Successive broods: an experiment to determine whether the expression or inheritance pattern of *psr* changes over successive broods of a female.
3. Lineage: an experiment to characterize inheritance of *psr* over successive generations within a lineage.
4. Venereal transmission: an experiment to test whether venereal transmission of *psr* can occur between females which have mated with a *psr* male and a later wild-type male which mates with the same female.
5. Within-host infectivity: an experiment to test for transmission of *psr* between a *psr* brood and an uninfected brood when both develop together within the same host.

The next series of experiments investigated the relationship between egg fertilization and transmission of *psr*. These experiments utilized genetic and environmental variability of the sex ratio of Nasonia to test the fertilization hypothesis. Since females are derived from fertilized eggs and males from unfertilized eggs in this haplodiploid species, the female sex ratio (percentage of females) is also the percentage of fertilized eggs. It is well documented in Nasonia that individual wasps can vary their sex ratio under different environmental circumstances. For example, the first wasp to parasitize a host produces around 85% females, whereas the second wasp produces a male-biased sex ratio (WYLIE 1966; WERREN 1980a). These responses are consistent with sex-ratio theory (WERREN 1980a, CHARNOV 1982). Other individual responses include a declining sex ratio with increasing wasp density (WERREN 1983) and a declining sex ratio immediately after copulation, apparently due to a temporary inability of females to utilize sperm (VAN DEN ASSEM and FEUTH DE BRUIJN 1977). In addition, there are strain differences in sex ratio, such as *msr* (~97% female) and sel (selected for a low female sex ratio; PARKER and ORZACK 1985). The fertilization hypothesis predicts that percentage of transmission of *psr* to the all-male brood will vary directly with the percentage of fertilized eggs (female sex ratio) under these different conditions and utilizing different stocks.

6. Strain transmission: an experiment to compare the percentage of transmission of *psr* to the percentage of fertilized eggs in three very different strains: Lab II, which produces around 85% fertilized eggs under the experimental conditions; sel, which

produces around 60% fertilized eggs; and *msr*, which produces around 97% fertilized eggs.

7. Male sperm depletion: an experiment to characterize the transmission of *psr* in males which have been successively mated to many females in rapid succession, such that sperm transfer is reduced.

8. Double matings: experiments that investigate the dynamics of *psr* transmission in females which have been mated to both *psr* and non-*psr* males.

Statistics: Statistical significance is computed using the Mann Whitney U test, Friedman's test, Chi square test or Fisher's exact test (SIEGEL 1956). For statistical analyses, individual broods rather than individual progeny are used as independent observations. This approach is more conservative and also correct, since the sexes of individual progeny within a brood are not necessarily statistically independent.

RESULTS

The first series of experiments characterize the basic inheritance pattern of the *psr* factor. Preliminary results revealed that although the *psr* factor causes the production of all-male broods, not all of the males in the brood express the *psr* characteristic. Therefore, the total brood experiment was conducted to determine what proportion of males in a *psr* family express the *psr* trait. Female (cb^+) wasps were individually mated to *psr* males, and each was then allowed to parasitize a *S. bullata* host for 24 hr. All the resulting males from each female were then tested for the *psr* trait by mating them individually to cb^+ females and observing the resulting sex ratio. As a control, cb^+ females were individually mated to cb^+ males. Two interesting findings are evident. First, 0.78 ± 0.07 SD ($N = 8$) proportion of males from *psr* broods express the *psr* trait. By comparison, the sex ratio (proportion of females) produced in the controls is $0.80 + 0.12$ SD ($N = 38$). The result suggested that transmission and expression of *psr* may be linked to fertilization of eggs by sperm. The second interesting finding is reflected in the distribution of sex ratios between males from *psr vs.* control broods shown in Figure 1. Approximately 78% of *psr* males produce all-male broods, and approximately 5% produce broods including less than a 0.10 proportion females. The remaining broods show a sex-ratio distribution similar to that of the controls. In contrast, among the controls only 5% produce all-male broods (due to inadequate matings), and 0% fall into the less than 0.10 category. The majority of broods have strongly female-biased sex ratios of between 0.7 and 1.0 proportion females. There is a highly significant difference in the two distributions ($\chi^2 = 198$, $P < 0.001$, d.f. = 11).

Although 22% of *psr* males did not express the all-male trait, these males may have inherited the *psr* factor and passed it on to their sons, who may themselves express the *psr* trait. To test this possibility, males from broods of different sex-ratio categories were tested for *psr* by individual mating to control females. The categories utilized were 0, 0–10, 10–70 and 70–100% female sex ratios. Of these males, 13 of 20, 14 of 18, 0 of 20 and 0 of 19, respectively, produced all-male broods. Between two and five males were taken from each family for the test. However, this was not recorded; therefore, family transmission frequencies cannot be determined for the data. Nevertheless, assuming independence, the frequency of *psr* expression from all-male and nearly all-

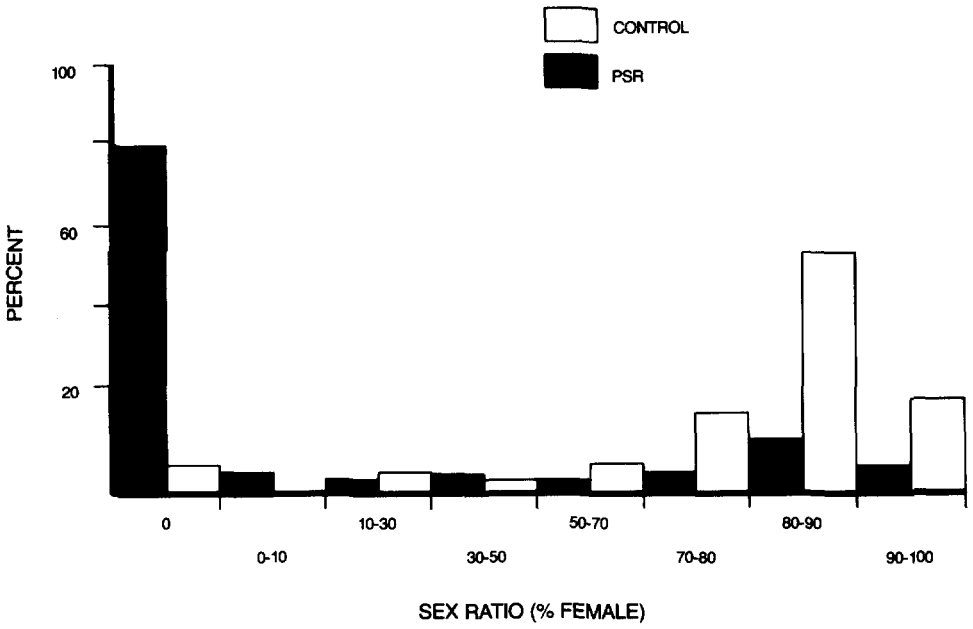

FIGURE 1.—The distribution of sex ratios produced by males from *psr* broods and control broods. Two hundred thirteen *psr* males from eight broods and 92 control males from 38 broods were tested.

male broods are significantly different from the mixed sex-ratio broods ($P < 0.0001$, Fisher's exact test).

Approximately 5% of males from *psr* broods produced sex ratios greater than 90% male but less than 100% males. Male progeny from these families actively transmitted the *psr* trait. In contrast, results show that, if a male from a *psr* brood reverts to producing a normal female-biased sex ratio, his sons do not express *psr* and, themselves, also produce normal sex ratios. Sample sizes are not large enough, however, to determine whether a low level of expression of *psr* occurs in the broods that have reverted to normal female-biased sex ratios.

Although males from broods that revert to normal do not express the *psr* trait, it is possible that they still transmit the character in a quiescent state, to be expressed in subsequent generations. The lineage experiment was designed to follow *psr* lineages over several generations to determine how expression of the trait varies over successive generations. Twenty-five lineages were established from the *psr* stock. Each generation, one male from each lineage was mated to a cb^+ female. The female was then given one *S. bullata* host for 24 hr, then two hosts for an additional 48 hr. Control cb^+ lineages were similarly treated. The experiment was conducted for ten generations. Five lineages were not successfully maintained for all ten generations and are excluded from analysis. Results are shown in Figure 2 and Table 1. By the seventh generation, all of the lineages had reverted to normal female-biased sex ratios. Apparently, once a lineage has lost expression of *psr*, the trait is not regained. Per gener-

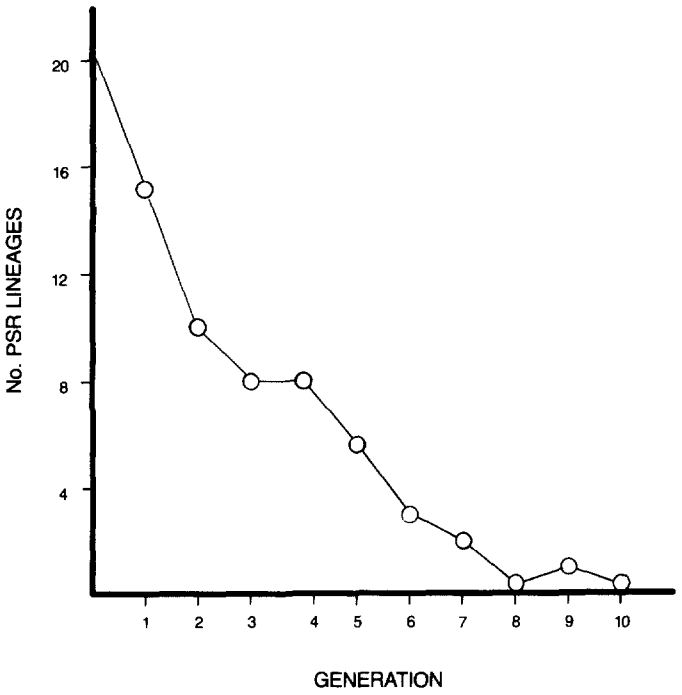

FIGURE 2.—The number of lineages producing high male sex ratios as a function of generation. All lineages were initially started from *psr* broods and were maintained from one male per generation.

TABLE 1

Integeneration transmission probabilities for *psr* and control lineages

Generation T	Lineages	Generation T + 1: High male sex ratio	Generation T + 1: Normal sex ratio	*N*
High male sex ratio	*psr*	0.60	0.40	52
	Control	0	1.0	4
Normal sex ratio	*psr*	0.05	0.95	127
	Control	0.06	0.94	64

ation transitional probabilities for *psr* and control lineages are shown in Table 1. High male broods are defined as those with sex ratios greater than 90% male; normal sex-ratio broods are those with sex ratios less than 90%. For *psr* lineages, there was a 0.60 probability of remaining mostly male in the next generation, and a 0.40 probability of reverting to normal sex ratio. Once producing normal sex ratios, the probability of going from normal to mostly male was 0.05, which does not differ significantly from the control probability of 0.06 ($P > 0.60$, $\chi^2 = 0.2$, d.f. = 1). Since it was possible that these "revertant" broods did return to *psr* expression, ten males from each of these

three broods were tested for *psr* in the usual fashion. None produced mostly male sex ratios, indicating that they had not returned to *psr* expression. Most likely these "revertants" resulted from inadequate mating, as occurs in 5% of control matings. One of the revertant lineages was maintained in culture for ten more generations, and 20 males from the culture were tested for *psr* expression on generation 15 and 20. None showed expression of *psr*. Thus, it can be tentatively concluded that once a *psr* lineage converts to normal sex ratio, the *psr* trait is at least temporarily, and probably permanently, lost. Similar lineage experiments have been repeated with the same general result that *psr* lineages decline in frequency over successive generations. This decline is due to the fact that only one male is used to maintain the lineage each generation. Since there is a 15–40% probability that any male has lost *psr*, it is inevitable that a lineage will eventually lose the *psr* trait when maintained in this fashion.

Both of the experiments above investigated *psr* expression and transmission in the first reproductive days of a female. Female wasps can live up to a month and produce up to 800 offspring in their lifetimes (WHITING 1967; VELTHUIS, VELTHUIS-KLUPPELL and BOSSINK 1965). Transmission and expression of *psr* may change over the life of a female, and the successive brood experiment tested for such temporal changes. Lab II females were mated to *psr* males and then were provided with four hosts per day for 13 days. Resulting progeny were scored for sex, and ten males per brood were tested for *psr*. Lab II × Lab II matings were used for a control. Data are presented in Figure 3. No significant changes were found in either expression or transmission of *psr* over the 13 days (Friedman's $\chi^2 = 2.2$, $P > 0.7$, d.f. = 4). In support of the fertilization hypothesis, there were no significant differences between percentage of transmission of *psr* and female sex ratio in the controls (Friedman's $\chi^2 = 2.7$, $P < 0.5$, d.f. = 4). Nor was the overall percentage of females (85.7, $N = 55$) significantly different from the overall percentage of *psr* transmission (86.5, $N = 145$, $U = 3705.5$, $z = 0.779$, $P > 0.4$).

Two preliminary experiments tested for infectious transmission of *psr*. The sonkiller factor, *sk*, is known to be infectiously transmitted within a host between *sk* offspring and "normal" offspring when both develop within the same host (SKINNER 1983; HUGER, SKINNER and WERREN 1985; WERREN, SKINNER and HUGER 1986). Similar transmission of *psr* within the host was tested for by mating Lab II females to *psr* males, and then allowing these females to parasitize a host for 48 hr. During the same period a virgin st-1 female had access to the same host. The use of virgin st-1 females allows identification of offspring from the *psr*-infected (Lab II) and noninfected (st-1) parent. Tests revealed no within-host infectivity. Forty-two st males were tested for *psr*, and none were found to be carriers. Two additional replicates have provided similar results.

The venereal transmission experiment tested for transmission of *psr* between males mating to a common female. *psr* males were individually mated to Lab II females, and males were removed after copulation but before postcopulatory courtship to facilitate second mating (VAN DEN ASSEM and VISSER 1976).

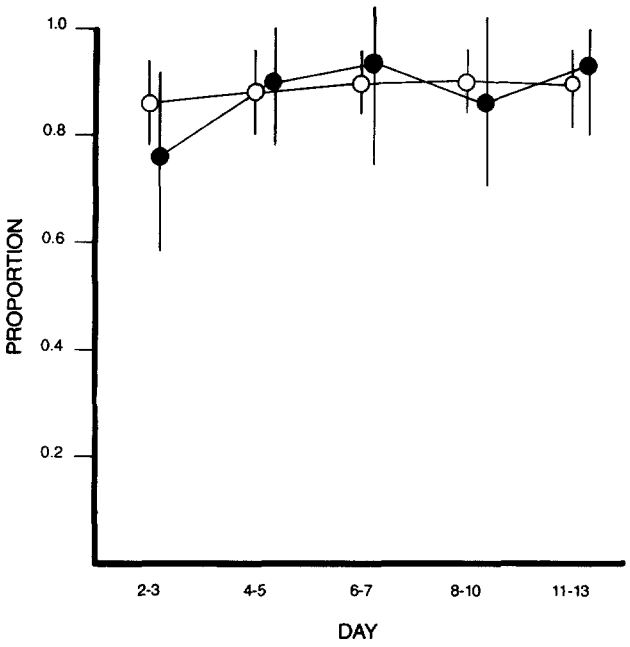

FIGURE 3.—Change in proportion females (O) in the control and proportion *psr* transmission (●) in *psr* broods over successive days of female oviposition. Standard deviations are shown. Data are from broods of 11 control females and 29 *psr*-mated females.

Twenty-four hours later the females were individually mated to Lab II males. These males were then tested for the *psr* trait by mating them to Lab II virgins. This particular experimental design would detect mechanical transmission of the *psr* factor through venereal contact. However, if the factor requires an incubation or amplification period of more than 24 hr before successful venereal transmission, the experiment would not detect it. Of the 52 males tested, none showed the *psr* trait.

The next series of experiments were designed to further test the fertilization hypothesis. As mentioned earlier, this hypothesis assumes that the *psr* factor is associated with sperm and is transmitted to eggs during fertilization. Since Nasonia can control egg fertilization and there is genetic variability in the character, the strain transmission experiments were conducted to see if *psr* transmission varied with differences in egg fertilization rate between strains. Two strains were tested. *msr* is a cytoplasmically inherited sex-ratio factor that produces a strongly female-biased sex ratio (~97%) by causing fertilization of an equally high proportion of its eggs (SKINNER 1982). sel is a stock that was artificially selected by PARKER and ORZACK (1985) for a less female-biased sex ratio than normally produced (WERREN 1983).

To test *msr*, *psr* males from a Lab II background were mated to either *msr* or Lab II females, which were then provided with one host for 24 hr and then with a new host for 24 hr. Control crosses (mating Lab II males to either Lab II or *msr* females) were also performed. Ten males from the second host

TABLE 2

Percentage of female and percentage of *psr* transmission for different strains

Strain	% female	% *psr*
msr	94.3 ± 7.3 (14)	94.3 ± 15.2 (11)
Lab II	70.7 ± 21.1 (24)	68.9 ± 14.8 (12)
sel	65.4 ± 11.5 (12)	67.5 ± 15.7 (15)
Lab II	85.7 ± 10.7 (15)	84.5 ± 15.0 (14)

Sample sizes (in parentheses) represent number of families tested.

of each *psr* brood were tested for *psr*. Table 2 compares the percentage of *psr* transmission in these tests to the percentage of females in the control crosses. Consistent with the fertilization hypothesis, *psr* transmission was significantly different in the two strains ($U = 12$, $P < 0.01$), but was not significantly different from the female sex ratio (proportion fertilized eggs) produced by each respective strain (*msr* $U = 53$, $P > 0.1$; Lab II $U = 104.5$, $P > 0.1$). *psr* transmission and percentage of fertilized eggs were both around 95% for the *msr* strain, and were around 75% for the Lab II strain. Strain mating experiments show that, in the strain with a high proportion of fertilized eggs, there is high transmission of *psr*, and in the strain with a low proportion of fertilized eggs, there is low transmission of *psr*.

The sel strain was tested by provisioning females for 48 hr with four hosts, then giving two hosts for 24 hr. Fifteen males from each *psr* brood were tested for the trait. Results are shown in Table 2. In this experiment the sex ratio of the sel strain is significantly lower than for the Lab II strain (61.1 *vs.* 85.7, $U = 9$, $P < 0.001$). Similarly, *psr* transmission is significantly lower in the sel strain ($U = 28$, $P < 0.01$). In neither strain did *psr* transmission differ significantly from the percentage of females normally produced by that strain (sel $U = 79$, $P > 0.1$; Lab II $U = 96$, $P > 0.1$).These results are consistent with the fertilization hypothesis.

Nasonia males which are successively mated to many females will eventually be depleted of sperm. This depletion is reflected in a decreasing sex ratio (few daughters) produced by the mated females. The sperm depletion experiment was conducted to determine how sperm depletion affects transmission of *psr*. Either *psr* or control (Lab II) males were individually mated to 30 females in rapid succession. Each mating was observed, and the female was removed and a new female introduced immediately after copulation. Courtship and copulation typically take less than 1 min under these circumstances. Females were then provided with one host for 24 hr and two hosts for the next 24 hr. The sex ratio of each control-mated female was recorded. In addition, the sex ratio of every third *psr*-mated female was recorded, and ten males from each such mating were tested for *psr*.

Results are shown in Figure 4. Clearly, there is a strong correlation between depletion of sperm and loss of *psr* transmission. In later matings, control males provided fewer sperm to females as reflected in their reduced sex ratio, and

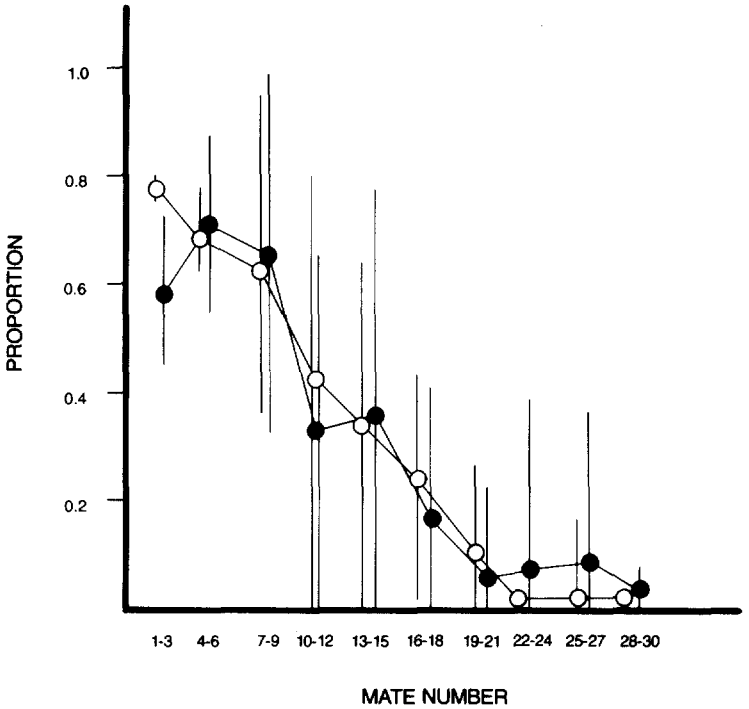

FIGURE 4.—Change in proportion females (○) of control and proportion *psr* transmission (●) in *psr* broods over successive male copulations to females. Standard deviations are shown.

psr transmission was similarly reduced. This result is consistent with the fertilization hypothesis; however, such a result is not inconsistent with the infection hypothesis. If a venereally infectious disease agent is associated with male ejaculate, then reduced male ejaculate could lower the concentration of the disease agent and, thus, the severity of the sex-ratio disease. However, the close quantitative fit between percentage of fertilized eggs and percentage of *psr* transmission is striking.

A final series of experiments provide even stronger evidence that the *psr* agent requires egg fertilization for transmission. When a female insect mates more than once, one mating may be more effective than the other. This is generally true in Nasonia. In multiple matings the first male's sperm is utilized predominantly by the female in fertilizing eggs. In addition, there is another very interesting effect of matings on sperm utilization. For 6–24 hr after mating, a Nasonia female utilizes no sperm and therefore produces only male offspring (unfertilized eggs) (VAN DEN ASSEM and FEUTH DE BRUIJN 1977). The sperm utilization phenomena can be used to dissect the relationship between egg fertilization and *psr* transmission.

Preliminary experiments revealed that some utilization of second male sperm is most likely to occur if the female is allowed to oviposit for 48 hr between matings (presumably, sperm have been used during this interval, and some vacant space is available in the spermatheca). Therefore, the following experimental design was utilized. Females were (1) individually mated (matings were

TABLE 3

Crosses performed in double-mating experiments

Female	First male	Second male	No. begun	No. completed
+/+	+	st	28	20
+/+	*psr*	st	24	15
+/+	+		10	8
+/+	*psr*		10	9
st/st	+	st	40	6
st/st	*psr*	st	18	2

TABLE 4

Percentage of female and percentage of *psr* transmission in double-mating experiments

Cross			% females		
♀	♂1	♂2	Before	During	After
+/+	+	st	76.3 ± 14.7 (20)	5.4 ± 21.6 (20)***	66.8 ± 19.6 (20)**
+/+	+		79.9 ± 7.1 (8)	91.5 ± 4.6 (7)	82.2 ± 14.2 (8)
+/+	*psr*	st	0.5 ± 0.9 (15)	0.0 ± 0.0 (15)	11.4 ± 7.2 (15)**
+/+	*psr*		0.8 ± 1.7 (8)	1.3 ± 2.9 (8)	1.6 ± 3.0 (8)
				% *psr* transmission	
+/+	*psr*	st	79.2 ± 11.3 (9)	6.6 ± 10.1 (8)***	49.8 ± 17.6 (8)***
+/+	*psr*		82.2 ± 11.6 (8)	87.4 ± 7.8 (8)	87.2 ± 3.2 (8)

Single-mating controls are indicated by a blank next to the second mating category. Significant differences between double mating and its control are based on the Mann Whitney U test (** $P < 0.01$; *** $P < 0.001$). Sample sizes (in parentheses) represent number of families tested.

observed) and presented with three hosts for 48 hr, (2) mated to a second male and presented with one host for 5 hr, and then (3) given six hosts for 48 hr. The three host presentation time periods are indicated as before, during and after, respectively, in reference to the sperm inhibition period following the second mating. Three genotypes were used: Lab II (+), *psr* in a Lab II genetic background (*psr*) and scarlet-eye mutant st-1 (st). The scarlet eye mutant is recessive to wild type. Recalling that females are diploid and males are haploid, the crosses shown in Table 3 were performed.

As shown, few of the st/st female double-mating crosses were successful. Observations revealed that few st/st females were willing to mate a second time, whereas 42 of 52 +/+ females copulated a second time. The number of "successful" *psr* × st/st crosses were further reduced because only a portion of the males from any *psr* brood typically carry the trait (72% in this experiment). Because of inadequate sample size, the st/st crosses are excluded from the analysis that follows.

Results, shown in Table 4 and Figure 5, clearly reveal that transmission of *psr* is strongly correlated with egg fertilization. A second copulation caused the sex ratio to drop significantly from 76.3 to 5.4% females ($U = 13$, $P < 0.001$) in the control crosses. The sex ratio then rebounded back to 66.8% (U

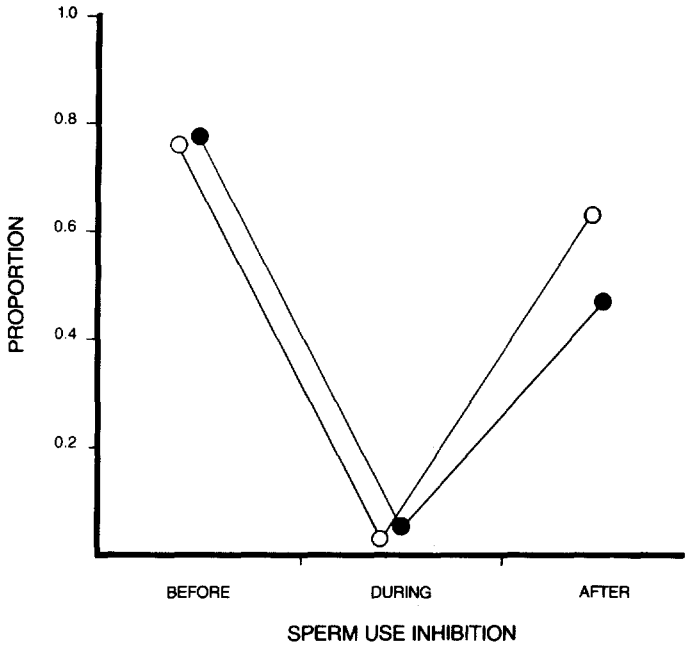

FIGURE 5.—Change in proportion females (○) of control and proportion *psr* transmission (●) in *psr* broods before, during and after the period of sperm-use inhibition following second matings.

= 17, $P < 0.001$). Similarly, a second copulation caused *psr* transmission to dramatically drop from 79.2 to 6.6% ($U = 0$, $P < 0.001$) and then rebound to 49.8% ($U = 1$, $P < 0.001$). This pattern strongly supports the fertilization hypothesis, since *psr* transmission varies with egg fertilization, even within a single individual.

Rare females were produced in some *psr* broods, as reflected by the 1% female sex ratio in +/+ × *psr* and +/+ × *psr* × st matings. The sex ratio in +/+ × *psr* × st matings significantly increased to 11.4% in the (after) time period following the second copulation (11.4 *vs.* 1.6, $U = 13$, $P < 0.01$), whereas there ws no such increase without a second mating. Why are a greater proportion of females produced subsequent to a second copulation? The effect is presumably due to some influence of (non-*psr*) st sperm or ejaculate. One version of the fertilization hypothesis is that the *psr* factor is tightly associated with *psr* sperm; therefore, utilization of some st sperm after the second copulation allows the production of more female offspring. If this hypothesis is true, then these females should be of the st/+ heterozygous genotype. A second hypothesis is that the *psr* factor is only loosely associated with sperm of *psr* males and has been diluted by the second copulation. If this hypothesis is correct, then the factor should associate and dissociate both from *psr* male and st male sperm. Female genotypes would then reflect the frequency of "+" and st sperm utilized in the spermatheca.

The two predictions were tested by taking 30 females from *psr* "after" broods and 30 females from control (+/+ × + × st) broods and determining

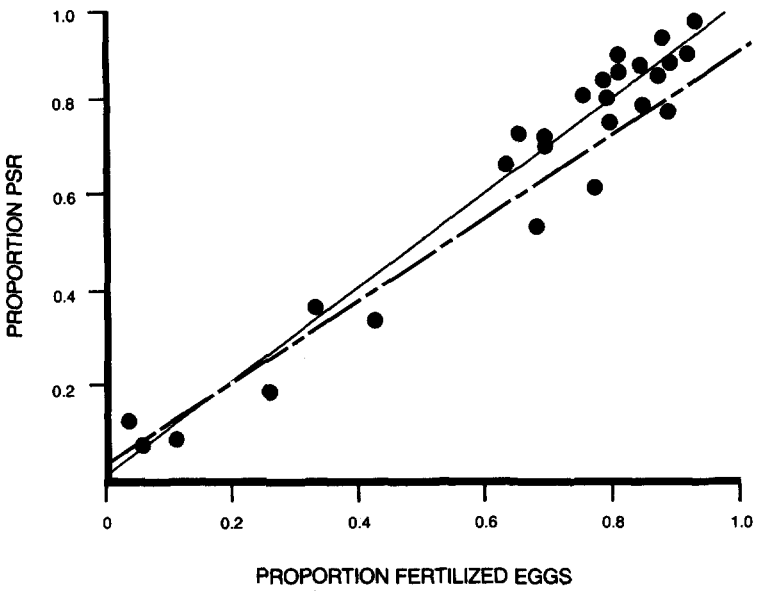

FIGURE 6.—Relationship between proportion fertilized eggs and proportion *psr* transmission.

their genotype by whether they produced only +, or both + and st eye-color, phenotype sons. All (30 of 30) females were heterozygous st/+ from the *psr* "after" broods, whereas only 6 of 30 females were heterozygous in the control broods. A precise statistical test for these data is difficult, because within-brood variance for the data was not determined. Assuming independence between individuals, the difference is significant ($P = 0.0015$, Fisher's exact test).

The pattern is also supported by a small data set from st/st female matings. These have the advantage that paternity can be determined in the F_1 because daughters from the *psr* males are phenotypically wild type (+/st), whereas a st father produces only st phenotype daughters (st/st). From the two successful st/st × *psr* × st double matings, one produced daughters in the "after" time period, and all 49 (100%) were "st." In contrast, in the six controls only 146 of 442 (33%) were scarlet eye. This shows that *psr* is not transmitted with st sperm in these crosses and, therefore, must be tightly associated with the *psr* male's sperm. Our (unpublished) studies have shown that st-*psr* males readily transmit and express *psr*: the result is not due to some inherent characteristic of st sperm. Rather, results show that the *psr* factor is tightly associated with the sperm of the *psr* males, and hence, daughters from double matings are predominantly produced by non-*psr* male sperm.

Results from the various experiments all support the fertilization hypothesis. These are summarized in Figure 6, which plots proportion fertilized eggs in controls vs. proportion *psr* transmission. Since there is variance in both the X and Y data points, results were regressed for both proportion *psr* transmission and proportion fertilized eggs as the independent variable, with both regressions shown on the plot. The weighted linear regression is highly significant for both lines ($y = 0.97x + 0.01$, $r^2 = 0.99$; $x = 1.18y - 0.03$, $r^2 = 0.96$) and

shows a strong linear relationship between *psr* transmission and egg fertilization. The average line between these two plots is $y = 0.91x + 0.02$.

The assay for *psr* transmission is slightly biased by the fact that a small proportion of normal, non-*psr* females also produce high male broods. These are females who either failed to mate or were not mated adequately. Therefore, some of the females producing high male sex ratios in matings with presumptive *psr* males may actually have been inadequately mated to non-*psr* males. Results from the total brood and lineage experiments show that 5% of matings in "normal" crosses resulted in high male sex ratios. Assuming that this probability is constant over the various experiments, then the actual *psr* transmission is estimated to be $P = 1.05h - 0.053$, where h = the proportion of high male sex-ratio broods in the assay, and P = actual transmission probability of *psr*. The data presented are not corrected for this small bias.

DISCUSSION

Results show that *psr* transmission is positively correlated with egg fertilization (1) between strains, (2) between successive mates of *psr* males and (3) within single individual females under different circumstances. Results also indicate that the factor is tightly associated with the sperm of *psr* males and is probably transmitted to the egg during the fertilization process. The mode of action of *psr* is, as yet, unknown. *psr* males are haploid and receive maternal chromosomes, but no paternal chromosomes (WERREN, SKINNER and CHARNOV 1981). Therefore, the *psr* factor must somehow prevent incorporation of the paternal chromosomes.

Cytoplasmic incompatibility causes rejection of the paternal pronucleus, resulting in male progeny, in crosses between certain strains of *N. vitripennis* (SAUL 1961). *psr* may prevent incorporation of the paternal genome in this fashion, or at a later or earlier time. For example, it is possible that *psr* prevents normal incorporation of chromosomes into the sperm during spermiogenesis of *psr* males, although electron microscopic examination has revealed no abnormalities during spermiogenesis of *psr* males (A. HUGER, personal communication). The mode of action of this element is under study.

The causative agent for *psr* is also unknown. Cytoplasmically inherited traits are often caused by organelles, or by intracellular microorganisms such as microsporidia, rickettsia, spiroplasms and viruses (GRUN 1976). For the sonkiller trait, histological examination readily revealed a bacteria as the causative agent (HUGER, SKINNER and WERREN 1985, WERREN, SKINNER and HUGER 1986); however, similar examination of *psr* males has so far not revealed an obvious causative agent (A. HUGER, personal communication). Such microorganisms can be difficult to find unless they are at high density, as was the sonkiller bacterium. Another possibility is that *psr* is a transposable element that is incorporated within the genome of *psr* males, but transposes before rejection of the paternal genome after egg fertilization. Since paternal extrachromosomal inheritance is so unusual, it is of great interest to determine the causative agent and mode of action of the *psr* factor.

The existence of rare females in *psr* broods may provide a clue to the

mechanism of *psr* transmission. Can such rare females transmit the *psr* factor? We have tested ten such females for *psr* transmission by testing their sons and daughters for expression of the trait. This preliminary study revealed no *psr* transmission; however, the phenomenon of rare females is currently under more thorough investigation (S. W. SKINNER, personal communication).

Population dynamics of the *psr* factor is expected to be strongly influenced by its transmission frequency. Since transmission of *psr* depends on egg fertilization by sperm, changes in *psr* frequency should be influenced by the sex ratio normally produced by Nasonia wasps. Simple panmictic models indicate that the *psr* factor can only exist in populations in which normal wasps produce a female-biased sex ratio (J. H. WERREN, unpublished results). In the experiments conducted here, transmission ranged from 0 to 94%, depending on the normal sex ratio. Since the wasp varies its sex ratio depending on local population structure (WYLIE 1966; WERREN 1980a,b, 1983), and nuclear and extranuclear variants in sex ratio exist (WERREN, SKINNER and CHARNOV 1981; WERREN, SKINNER and HUGER 1986; SKINNER 1982, 1983, 1985; PARKER and ORZACK 1985), such sources of sex-ratio variation are probably important in *psr* population dynamics.

Little is currently known about the population biology of *psr* in natural populations. The only field study of the factors so far conducted showed *psr* to occur at 7% in Utah populations, with *psr* at 17% and *sk* at 2% (SKINNER 1983). The frequencies of these factors in natural populations are likely to be affected by population structure. *N. vitripennis* has a highly demic population structure in nature (WERREN 1980, 1983; SKINNER 1983). Hosts are patchily distributed in bird nests and carcasses. Mating routinely occurs between flightless males and females immediately after wasp emergence in the local demes. Therefore, it is possible that both intrademic and interdemic selective forces affect frequency of the sex-ratio factors.

In addition, the *psr* factor is affected by, and has an effect on, frequency of the other two sex-ratio distorters. Since *psr* transmission is linked to egg fertilization, its frequency is enhanced by the presence of *msr*, which produces 97% fertilized eggs. However, *psr* negatively impacts upon the frequency of *msr*, since males from *psr* × *msr* matings do not transmit the maternally inherited *msr* to future generations. The sonkiller factor probably does not greatly enhance frequency of *psr* because it causes unfertilized egg lethality, rather than an increase in the number of fertilized eggs. By killing unfertilized eggs, sonkiller may decrease the degree of mate competition encountered by *psr* and thus, perhaps, increase its frequency. In contrast, matings between *psr* × *sk* result in *viable* haploid male offspring which are *psr* (WERREN, SKINNER and HUGER 1986). Therefore *psr* negatively impacts upon the frequency of *sk* in natural populations, since *sk* is transmitted through females which are eliminated in *sk* × *psr* matings. The *psr* factor also negatively affects chromosomal genes, since these are not transmitted to future generations by *psr* males. Selection could well favor autosomal modification and suppression of the *psr* factor. Clearly, the potential for "intragenomic conflict" in sex-ratio expression is great in this system.

It is currently difficult to say how this complicated sex-ratio system is maintained in natural populations. Simple models predict that the *msr* factor should go to, or near, fixation (LEWIS 1941; BULL 1983); however, this clearly has not occurred in Utah populations. Studies of different natural populations will help elucidate the impact of demic population structure on frequency of the sex-ratio distorters. Furthermore, there is no reason to believe that such sex-ratio factors are limited to Nasonia; therefore, surveys of other haplodiploid species are warranted. The Nasonia sex-ratio system appears to be well suited for studying interdemic selection in nature, because sex ratio is easily quantifiable, sex-ratio selection is known to be influenced by demic population structure (HAMILTON 1967; WILSON and COLWELL 1981; CHARNOV 1982) and demic population structure is characteristic of this wasp species (WERREN 1980b, 1983; SKINNER 1983).

We thank S. W. SKINNER and S. ORZACK for providing certain stocks. Thanks are extended to A. ORR and S. SKINNER, who gave comments on the manuscript, and to G. A. JEPPESEN, C. REMIE, S. W. SKINNER and H. VERBEEK, who provided assistance and advice in pilot studies and several experiments on the *psr* factor. ERIC CHARNOV kindly provided support for some of the pilot studies.

LITERATURE CITED

BIRKY, C. W., JR., 1976 The inheritance of genes in mitochondria and chloroplasts. Bioscience **26:** 26–33.

BULL, J. J., 1983 *Evolution of Sex-Determining Mechanisms.* Benjamin-Cummings, Menlo Park, California.

CHARNOV, E. L., 1982 *The Theory of Sex Allocation.* Princeton University Press, Princeton, New Jersey.

COSMIDES, L. M. and TOOBY, 1981 Cytoplasmic inheritance and intragenomic conflict. J. Theor. Biol. **89:** 83–129.

EBERHARD, W. G., 1980 Evolutionary consequences of intracellular organelle competition. Q. Rev. Biol. **55:** 231–249.

GRUN, P., 1976 Cytoplasmic genetics and evolution. Columbia University Press, New York.

HAMILTON, W. D., 1967 Extraordinary sex ratios. Science **156:** 477–488.

HUGER, A., S. W. SKINNER and J. H. WERREN, 1985 Bacterial infections associated with the son-killer trait in the parasitoid wasp *Nasonia* (=*Mormoniella*) *vitripennis*. J. Invertebr. Pathol. **46:** 272–285.

LEWIS, D., 1941 Male sterility in natural populations of hermaphroditic plants: the equilibrium between females and hermaphrodites to be expected with different types of inheritance. New Phytol. **40:** 56–63.

PARKER, E. D., JR. and S. H. ORZACK, 1985 Genetic variation for the sex ratio in *Nasonia vitripennis*. Genetics **110:** 93–105.

RYAN, S. L. and G. B. SAUL II, 1968 Post-fertilization effect of incompatibility factors in *Mormoniella*. Mol. Gen. Genet. **103:** 24–36.

SAUL, G. B., 1961 An analysis of non-reciprocal cross incompatibility in *Mormoniella vitripennis* (Walker). Z. Vererbungsl. **92:** 28–33.

SAUL, G. B., P. W. WHITING, S. W. SAUL and C. A. HEIDNER, 1965 Wild-type and mutant stocks in Mormoniella. Genetics **52:** 1317–1327.

SIEGEL, S., 1956 *Nonparametric Statistics for the Behavioral Sciences.* McGraw Hill, New York.

SKINNER, S. W., 1982 Maternally inherited sex ratio in the parasitoid wasp, *Nasonia vitripennis.* Science **215:** 1133–1134.

SKINNER, S. W., 1983 Extrachromosomal sex ratio factors in the parasitoid wasp, *Nasonia* (=*Mormoniella*) *vitripennis.* Ph.D. Dissertation, University of Utah, Salt Lake City.

SKINNER, S. W., 1985 Son-killer: a third extrachromosomal factor affecting sex ratio in the parasitoid wasp, *Nasonia* (=*Mormoniella*) *vitripennis.* Genetics **109:** 745–754.

UYENOYAMA, M. K. and M. W. FELDMAN, 1978 The genetics of sex ratio distortion by cytoplasmic infection under maternal and contagious transmission: an epidemiological study. Theor. Pop. Biol. **14:** 471–497.

VAN DEN ASSEM, J. and E. FEUTH DE BRUIJN, 1977 Second matings and their effect on the sex ratio of the offspring in *Nasonia vitripennis* (Hymenoptera: Pteromalidae). Entomol. Exp. Appl. **21:** 23–28.

VAN DEN ASSEM, J. and J. VISSER, 1976 Aspects of sexual receptivity in female *Nasonia vitripennis.* Biol. Comport. **1:** 37–56.

VELTHUIS, H. H., F. F. VELTHUIS-KLUPPELL and G. A. H. BOSSINK, 1965 Some aspects of the biology and population dynamics of *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae). Entomol. Exp. Appl. **8:** 205–227.

WERREN, J. H., 1980a Sex ratio adaptations to local mate competition in a parasitic wasp. Science **208:** 1157–1159.

WERREN, J. H., 1980b Studies in the evolution of sex ratios. Ph.D. Dissertation, University of Utah, Salt Lake City.

WERREN, J. H., 1983 Sex ratio evolution under local mate competition in a parasitic wasp. Evolution **37:** 116–124.

WERREN, J. H., S. W. SKINNER and E. L. CHARNOV, 1981 Paternal inheritance of a *daughterless* sex ratio factor. Nature **293:** 467–468.

WERREN, J. H., SKINNER, S. W. and A. HUGER, 1986 Male-killing bacteria in a parasitic wasp. Science **231:** 990–992.

WHITING, A. R., 1967 The biology of the parasitic wasp, *Mormoniella vitripennis.* Q. Rev. Biol. **43:** 333–406.

WILSON, D. S. and R. COLWELL, 1981 Evolution of sex ratio in structured demes. Evolution **35:** 882–897.

WYLIE, H. G., 1966 Some mechanisms that affect the sex ratio of *Nasonia vitripennis* (Walker) (Hymenoptera:Pteromalidae) reared from superparasitized house fly pupae. Can. Entomol. **98:** 645–653.

Communicating editor: D. CHARLESWORTH